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DEVELOPMENT OF NEW RP-HPLC METHOD FOR SIMULTAENOUS ESTIMATION OF AMILORIDE AND HYDROCHLOROTHIAZIDE

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Abstract:

A simple, Accurate, precise method was developed for the simultaneous estimation of the Amiloride and Hydrochlorthiazide in Tablet dosage form. Chromatogram was run through BDS (250mm 4.6mm, 5µ). Mobile phase containing Buffer and Acetonitrie in the ratio of 45:55A was pumped through column at a flow rate of 1ml/min. Temperature was maintained at 30°C. Optimized wavelength for Amiloride and Hydrochlorthiazide was 296nm. Retention time of Amiloride and Hydrochlorthiazide were found to be 4.081 min and 2.554 min. %RSD of the Amiloride and Hydrochlorthiazide were and found to be 0.57 and 0.71 respectively. %Recover was Obtained as 100.40% and 99.9% for Amiloride and Hydrochlorthiazide. LOD, LOQ values were obtained from regression equations of Amiloride and Hydrochlorthiazide were 0.06ppm, 0.14ppm and 0.18ppm, 0.41ppm respectively. Regression equation of Amiloride is y = 8186x + 1334, and of Hydrochlorthiazide is y = 3525x + 408.9.

Key Words: *Amiloride, Hydrochlorthiazide, RP-HPLC*

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1. INTRODUCTION:

Introduction to HPLC

The phenomenal growth in chromatography is largely due to the introduction of the versatile technique called high-pressure liquid chromatography, which is frequently called high-performance liquid chromatography. Both terms can be abbreviated as HPLC.[7] High-pressure liquid-solid chromatography (HPLC) is rapidly becoming the method of choice for separations and analysis in many areas. Most of the samples that are dissolved can be separated on some type of HPLC column.[1-4]

Characteristics of HPLC method[5-7]

- Efficient, highly selective, widely applicable
- Only small sample required.
- May be nondestructive of sample
- Easily flexible to quantitative analysis.
- High resolving power.

Modes of HPLC: [8-10]

1) Normal phase chromatography: The nature of stationary phase is polar and the mobile phase is non-polar in this mode .In this technique, non-polar compounds travel faster and are eluted first because of the lower affinity between the non-polar compounds and stationary phase. The time for polar compounds to elute takes longer time because of their higher affinity to the stationary phase, therefore generally this method is not used in the pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

2) Reversed phase chromatography: Reversed phase mode is the most popular mode for analytical and preparative separations of compounds of concern in biologicalproducts, pharmaceutical formulations & API's, chemical subastances, food and biomedical engineering. The stationary phase is non-polar hydrophobic packing with octyl and octadecyl functional group bonded to silica gel and the mobile phase is a polar solvent, often a partially or fully aqueous mobile phase. Polar substances prefer the mobile phase and elute first. As the hydrophobic character of the solutes increases, retention increases. Generally, the lower the polarity of the mobile phase, higher is the eluent strength.

2. DRUG PROFILE

2.1. Hydrochlorothiazide

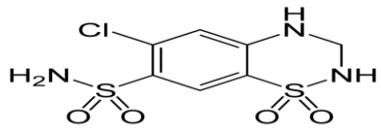

Fig-1-Strcture of Hydrochlorothiazide (HCTZ)

Application : A carbonic anhydrase inhibitor
Purity : ≥98%
Molecular Weight : 297.74
Molecular Formula : $C_7H_8ClN_3O_4S_2$
Physical State : solid
Solubility : Soluble in Methanol
Storage : Store at room temperature
Melting Point : 273-277°C (lit.)
P^{ka} : 7.9

2.2. Amiloride

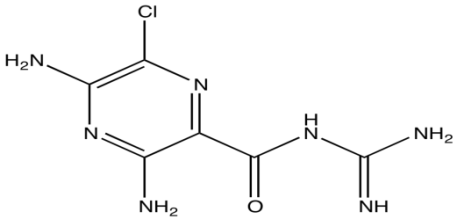

Fig -2- Structure of Amiloride

Synonym : N-Amidino-3,5-diamino-6-chloropyrazinecarboxamide hydrochloride
Application : A calcium channel and sodium channel protein inhibitor
Purity : ≥98%
Molecular Weight : 299.76
Molecular Formula : C11H18ClN7O
Physical State : Solid
Solubility : Soluble in water (partly), 0.1N HCl, methanol (9.80 - 10.20 mg/ml), DMSO, dilute aqueous acid, DMF, and acetone.
Storage : Store at 4° C
Melting Point : 202-205 °C (lit.) (tautomer)
Boiling Point : 543.95 °C at 760 mmHg (Predicted)
Density : 1.50 g/cm3 (Predicted)
Refractive Index : n20D 1.67 (Predicted)
pKa : 7.82 (Predicted)
pKb : 3.71 (Predicted)

3. MATERIALS AND METHODS:

3.1. Materials Used

Amiloride and Hydrochlorthiazide, acetonitrile, phosphate buffer, ammonium acetate buffer, glacial acitic acid, methanol, potassium dihydrogen phosphate buffer, tetra hydrofuran, tri ethyl amine, ortho-phosphoric acid etc.

3.2. Method Development: There are many trials were done by changing columns and Mobile phases and were reported below[11-16]

Trial 1: This trial was run through ODS 250 column with mobile phase composition of 50:50A KH_2PO_4 Buffer and Acetonitrile, Flow rate set at 1ml/min.

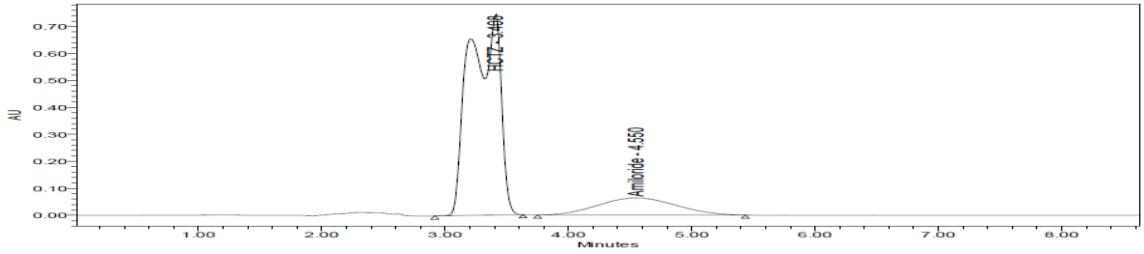

Fig-3- Trial chromatogram 1

Observation: peak splitting was observed.

Trial 2: This trial was run through Altima 150mm column with mobile phase composition of 50: 50 OPA Buffer and Acetonitrile, Flow rate set at 0.8ml/min.

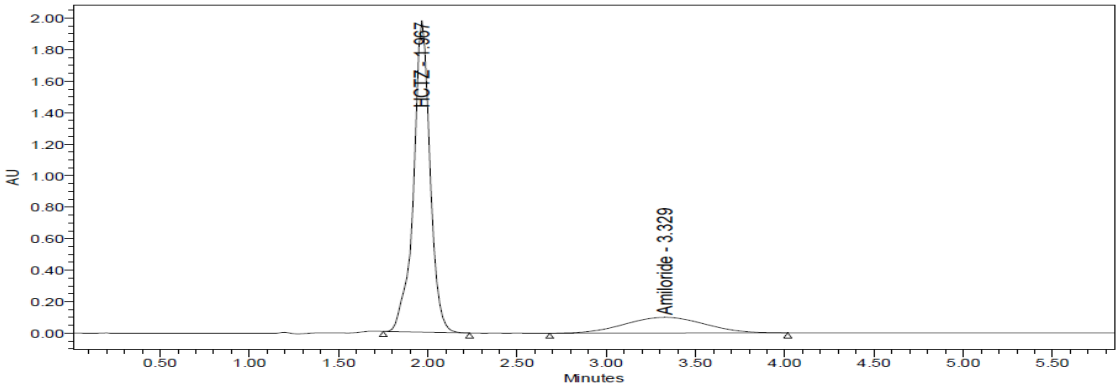

Fig -4- Trial chromatogram 2

Observation: Peaks eluted in void volume.

Trial 3: This trial was run through BDS 250mm column with mobile phase composition of 50:50 OPA Buffer and Acetonitrile, Flow rate set at 1ml/min.

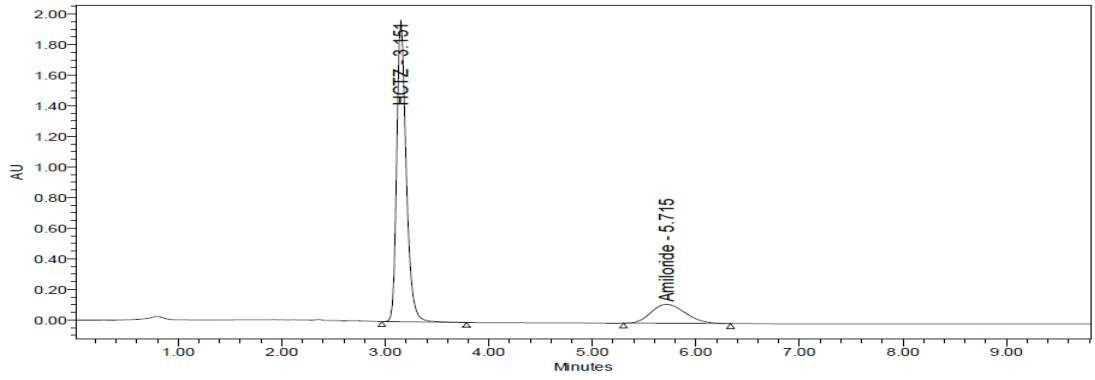

Fig-5- Trial chromatogram 3

Observation: In HCTZ peak fronting is observed.

Trial 4: This trial was run through BDS 250mm column with mobile phase composition of 45:45 0.02N NA2PO4 Buffer and Acetonitrile, Flow rate set at 1 ml/min.

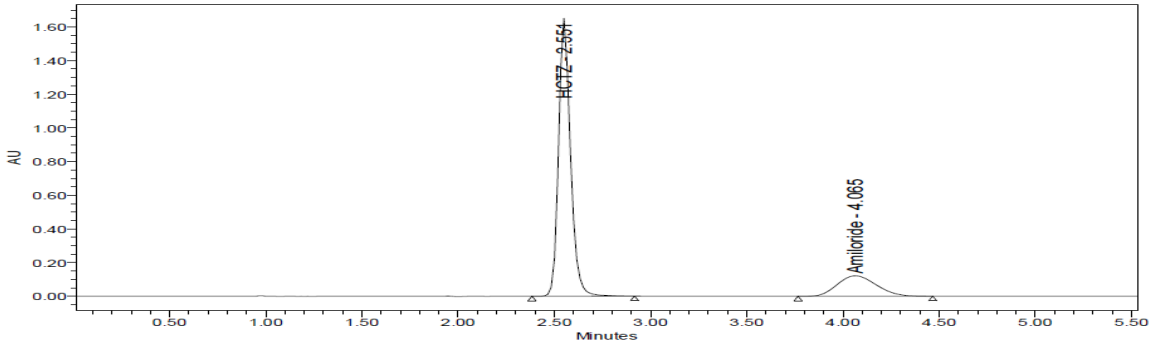

Fig-6-Trial chromatogram 4

Observation: both are eluted with good results.

Optimized Method: Drugs were eluted with good resolution, retention time all the parameters like Plate count and Tailing factor were within the limits.

Mobile phase:
Buffer and Acetonitrile taken in the ratio 45:55A

Chromatographic conditions:

Flow rate	**:**	1ml/min
Column	**:**	BDS 250 x 4.6 mm, 5µ.
Detector wave length	**:**	296nm
Column temperature	**:**	30°C
Injection volume	**:**	10µL
Run time	**:**	20min
Diluent	:	First dissolved in methanol and diluted with buffer

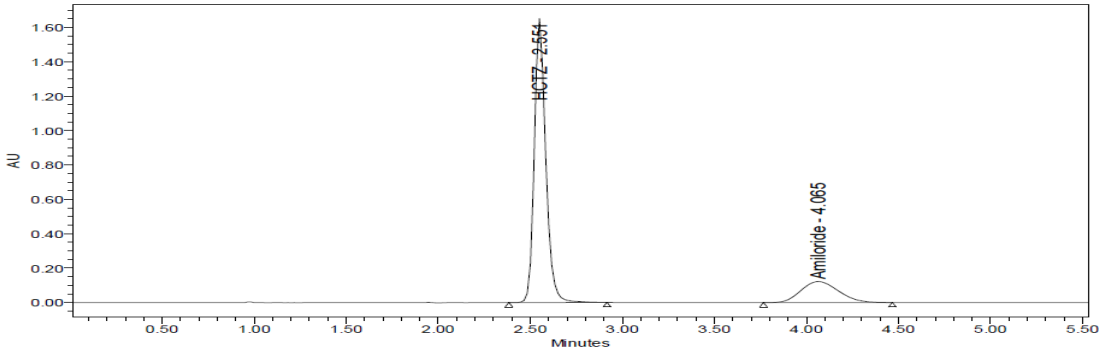

Fig-7-Optimized chromatogram of Amiloride and Hydrochlorthiazide

4. RESULTS AND DISCUSSIONS

1. **Systemsuitability:** All the system suitability parameters are within range and satisfactory as per ICH guidelines

Table-1- System suitability studies of Amiloride and Hydrochlorthiazide method

Property	Amiloride	Hydrochlorthiazide
Retention time (t_R)	4.081± 0.3 min	2.554±0.3min
Theoretical plates (N)	3841 ± 163.48	7944± 163.48
Tailing factor (T)	1.14 ± 0.117	1.18± 0.117

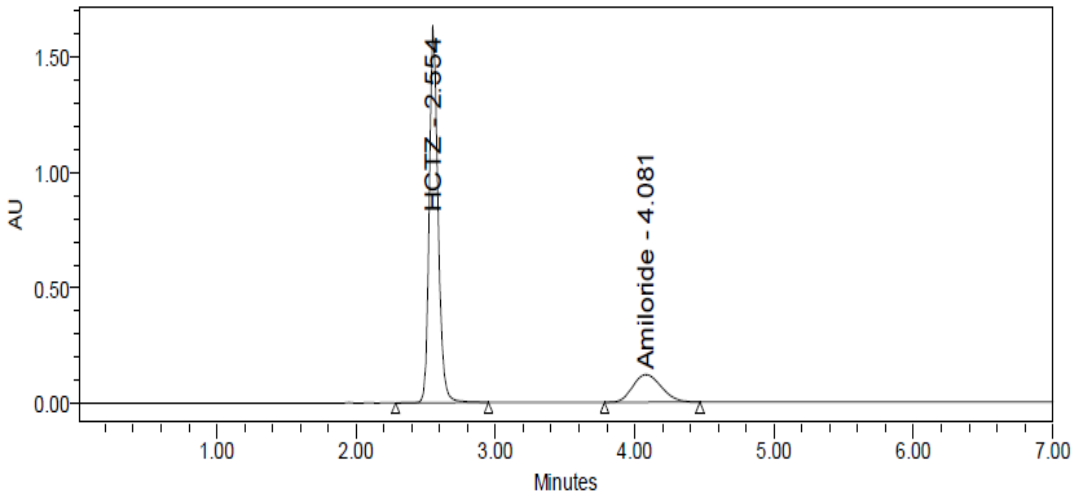

Fig-8-Typical chromatogram of Amiloride and Hydrochlorthiazide.

Linearity: Six Linear concentrations of Amiloride(5-30ppm) and Hydrochlorthiazide (50ppm to 300ppm) are prepared and Injected. Regression equation of Amiloride is y = 8186x + 1334, and of Hydrochlorthiazide is y = 3525x + 408.9 and the regression co-efficient was 0.999.

Table-2-Calibration data of Amiloride and Hydrochlorthiazide method.

S.no	Concentration Amiloride (µg/ml)	Response	Concentration Hydrochlorthiazide (µg/ml)	Response
1	0	0	0	0
2	5	43079	50	180488
3	10	84948	100	342047
4	15	124954	150	534232
5	20	161251	200	708457
6	25	208097	250	887143
7	30	246587	300	1051771

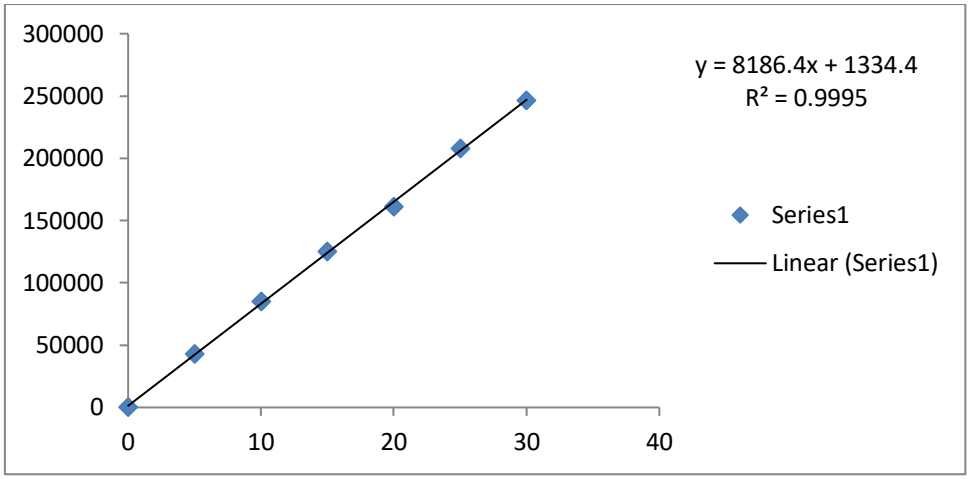

Fig-9-Calibration curve of Amiloride

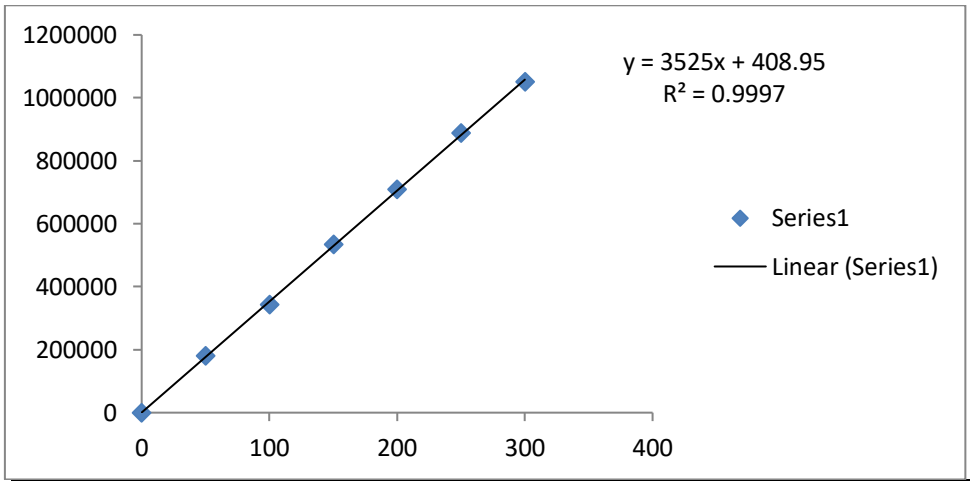

Fig-10- Calibration curve of Hydrochlorthiazide

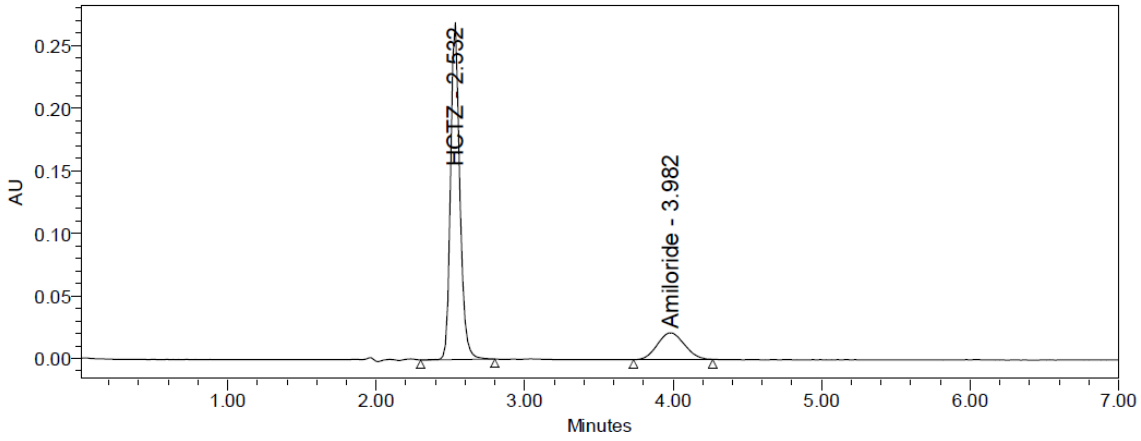

Fig-11- Linearity 25% Chromatogram of Amiloride and Hydrochlorthiazide method.

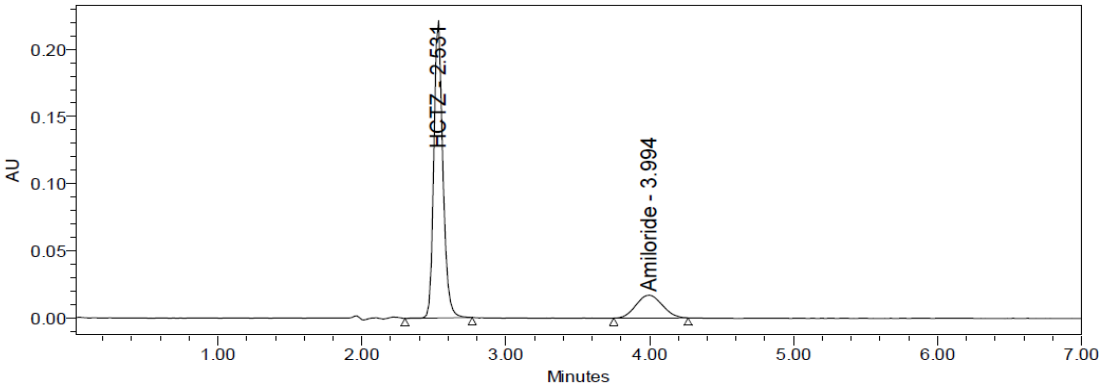

Fig-12- Linearity 50% Chromatogram of Amiloride and Hydrochlorthiazide method.

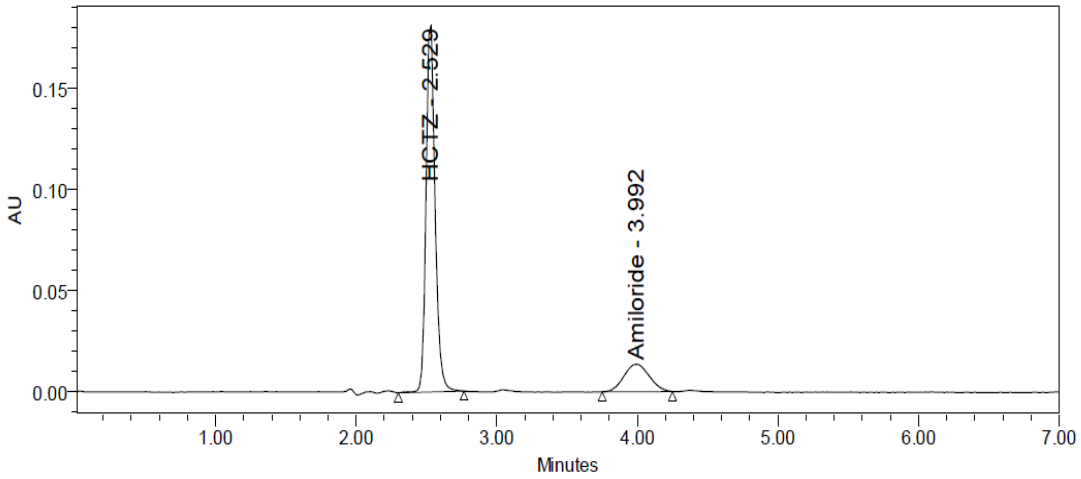

Fig-13- Linearity 75% Chromatogram of Amiloride and Hydrochlorthiazide method.

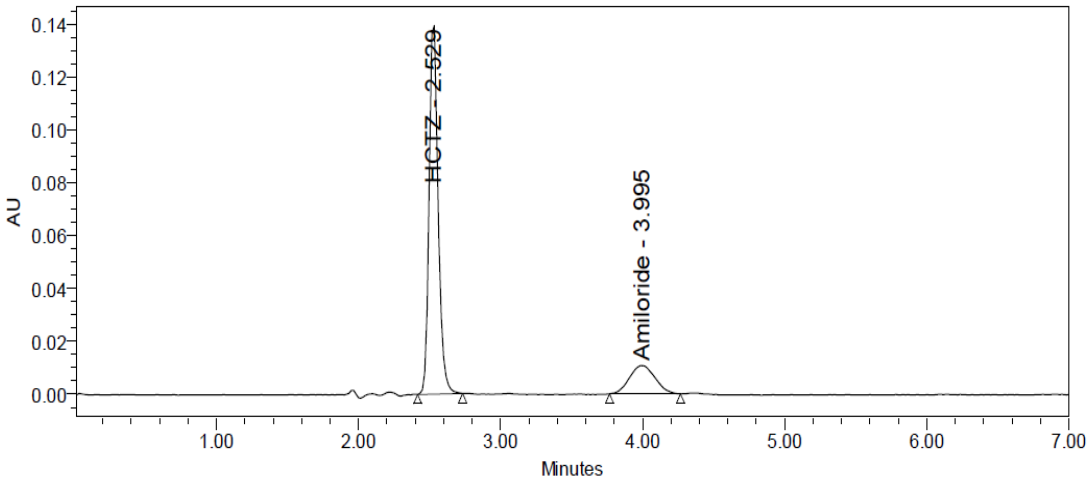

Fig-14- Linearity 100% Chromatogram of Amiloride and Hydrochlorthiazide method.

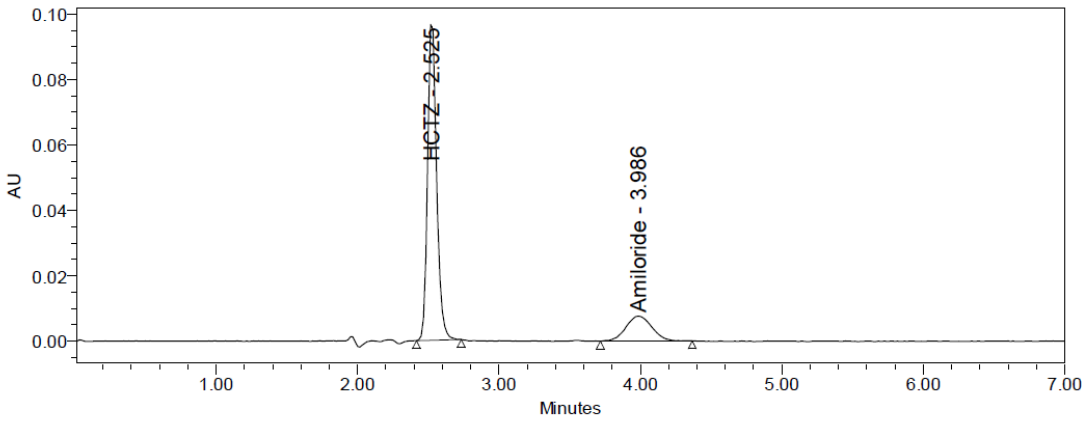

Fig-15-Linearity 125% Chromatogram of Amiloride and Hydrochlorthiazide method.

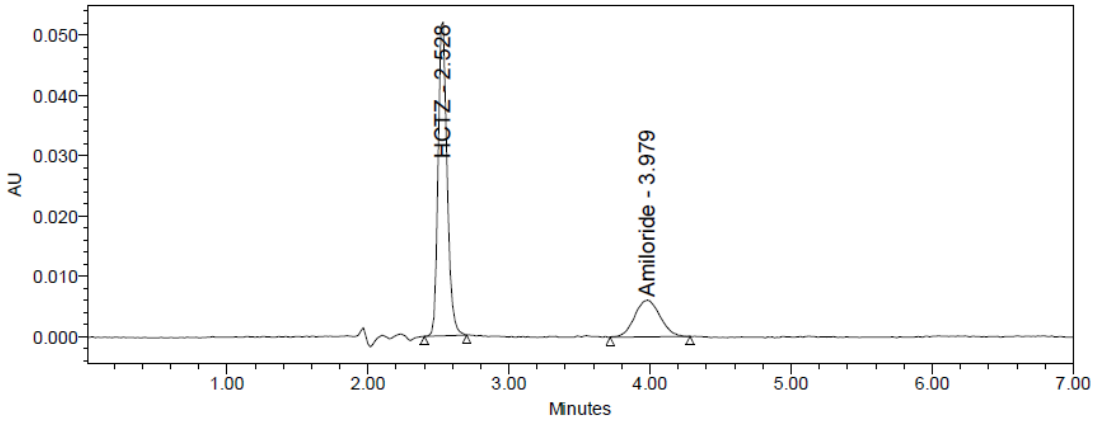

Fig-16- Linearity 150% Chromatogram of Amiloride and Hydrochlorthiazide method.

2. Precision:

Intraday precision (Repeatability): Intraday Precision was performed and % RSD for Amiloride and Hydrochlorthiazide were found to be 0.57% and 0.5% respectively.

Table-3-Repeatability results for Amiloride and Hydrochlorthiazide .

Sr. No.	Amiloride	Hydrochlorthiazide
1	1603523	7228177
2	1619954	7161044
3	1617499	7214508
4	1627720	7147013
5	1620588	7138384
6	1629622	7161716
Mean	1619818	7175140
Std. Dev.	9274	37104.7
%RSD	0.57	0.5

*Average of six determinations

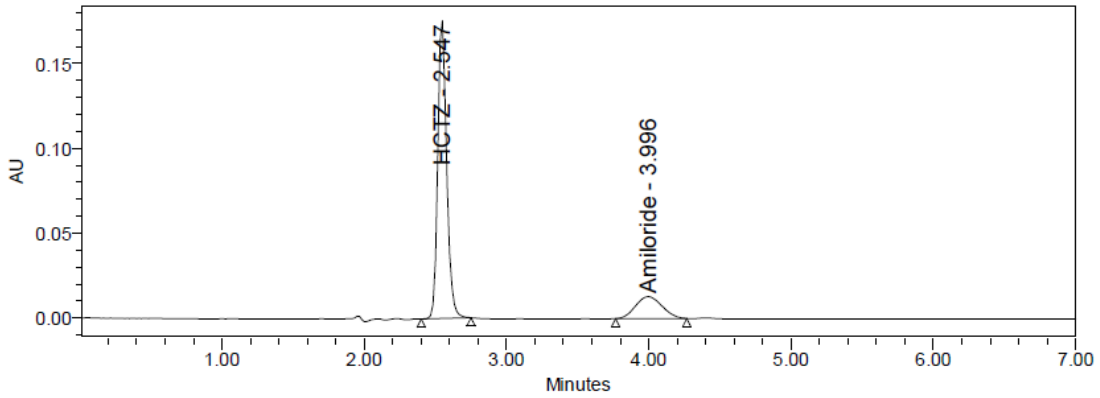

Fig-17- Repeatability Chromatogram of Amiloride and Hydrochlorthiazide method.

Inter day precision: Inter day precision was performed with 24 hrs time lag and the %RSD Obtained for Amiloride and Hydrochlorthiazide were 1.14% and 1.0%.

Table-4- Inter day precision results for Amiloride and Hydrochlorthiazide

Sr. No.	Amiloride	Hydrochlorthiazide
1	180777	779771
2	182967	783074
3	183477	768668
4	180642	764183
5	185663	771585
Mean	182705	773456
Std. Dev.	2085	7823
%RSD	1.14	1.0

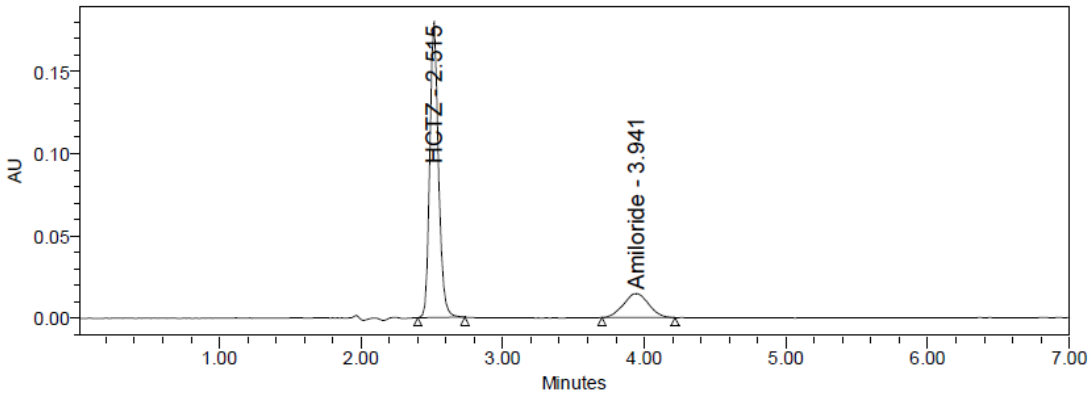

Fig-18- Inter Day precision Chromatogram of Amiloride and Hydrochlorthiazide method.

3. **Accuracy:** Three concentrations 50%, 100%, 150%, were injected in a triplicate manner and amount Recovered and % Recovery were displayed in Table 5.

Table-5- Accuracy results of Amiloride and Hydrochlorthiazide

Sample	Amount added (µg/ml)	Amount Recovered (µg/ml)	Recovery (%)
	10	9.981554	99.82
	20	20.09305	100.47
	30	30.11817	100.39
	100	99.35899	99.36
	200	200.7152	100.36
Hydrochlorthiazide	300	300.5042	100.17

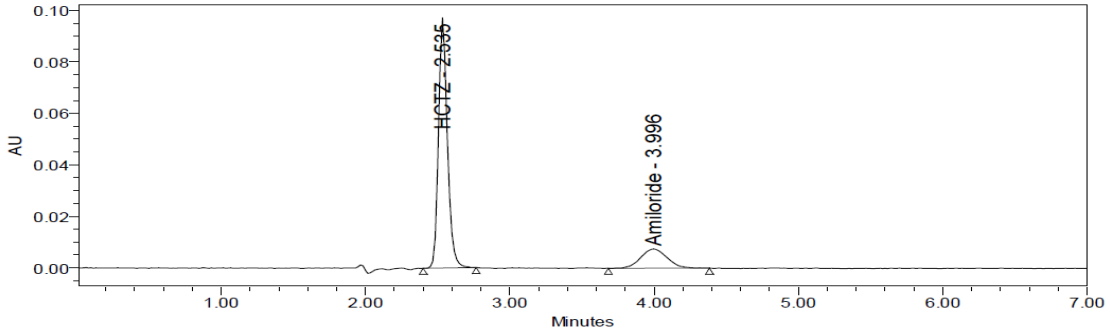

Fig-19- Accuracy 50% Chromatogram of Amiloride and Hydrochlorthiazide method.

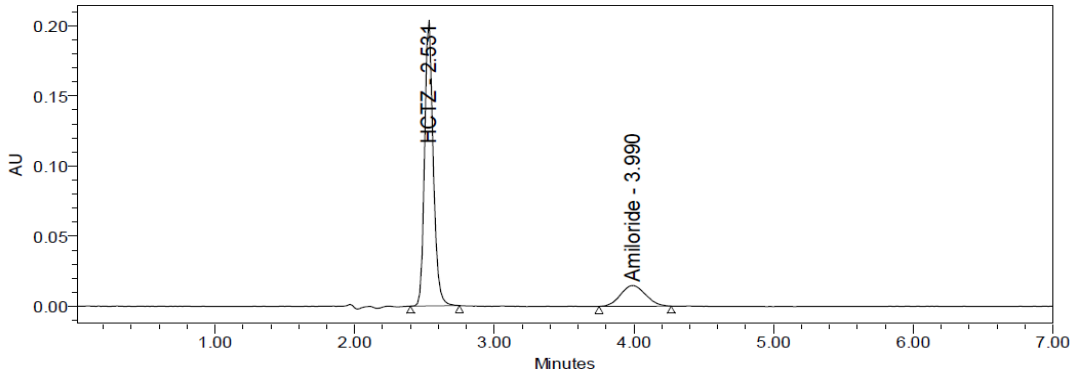

Fig-20- Accuracy 100% Chromatogram of Amiloride and Hydrochlorthiazide method.

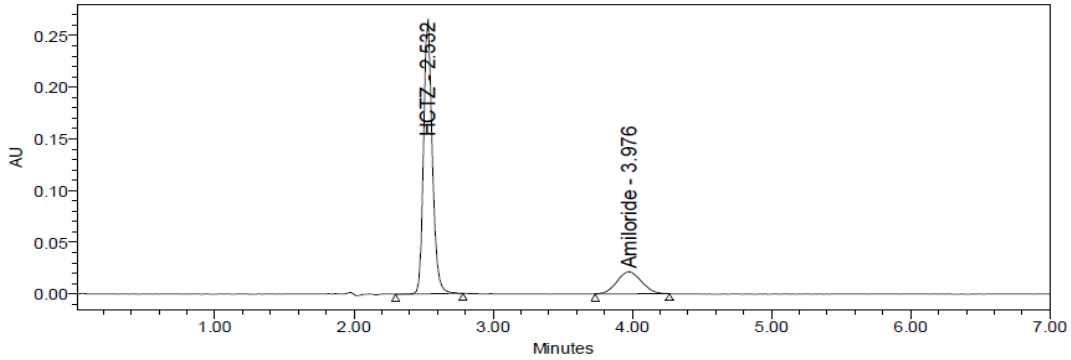

Fig-21-Accuracy 150% Chromatogram of Amiloride and Hydrochlorthiazide method.

4. **LOD:** Limit of ditection was calculated by inteAmiloride and Hydrochlorthiazidept method and LOD for Amiloride was found to be 0.06 and Hydrochlorthiazide was 0.14 respectively.

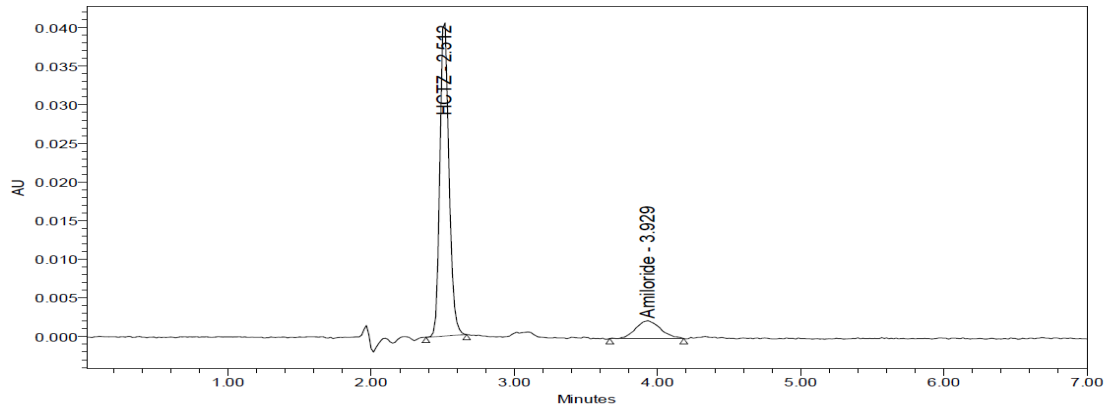

Fig-22-LOD Chromatogram of Amiloride and Hydrochlorthiazide method.

5. **LOQ:** Limit of Quantification was calculated by inteAmiloride and Hydrochlorthiazidept method and LOQ for Amiloride and Hydrochlorthiazide wre found to be 0.18 and 0.41 respectively.

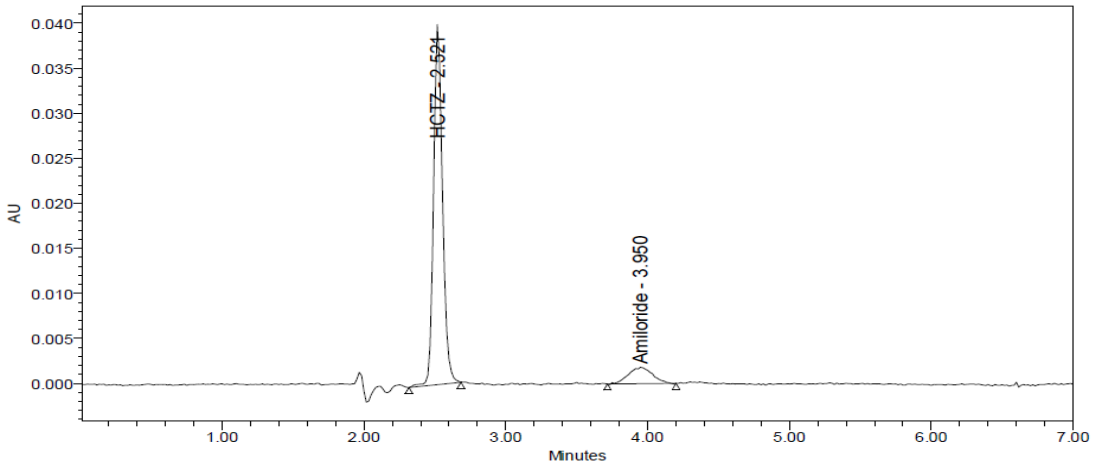

Fig-23- LOQ Chromatogram of Amiloride and Hydrochlorthiazide method.

6. **Robustness:** Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines.

Table -6- Robustness data of Amiloride and Hydrochlorthiazide method.

S.NO	Robustness condition	Amiloride %RSD	Hydrochlorthiazide %RSD
1	Flow minus	0.6	1.4
2	Flow Plus	1.3	0.8
3	Mobile phase minus	1.3	0.4
4	Mobile phase Plus	0.3	0.3
5	Temperature minus	0.1	0.3
6	Temperature Plus	0.4	0.4

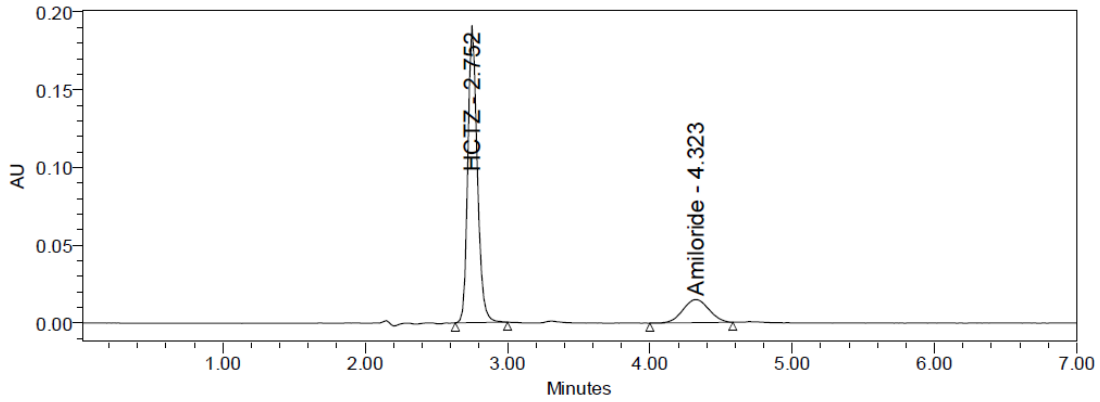

Fig-24- Flow minus Chromatogram of Amiloride and Hydrochlorthiazide method.

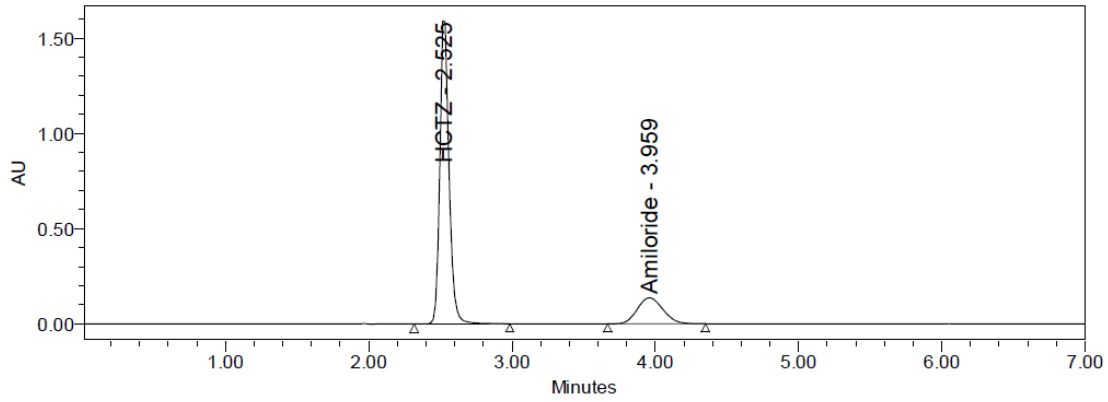

Fig-25- Flow plus Chromatogram of Amiloride and Hydrochlorthiazide method.

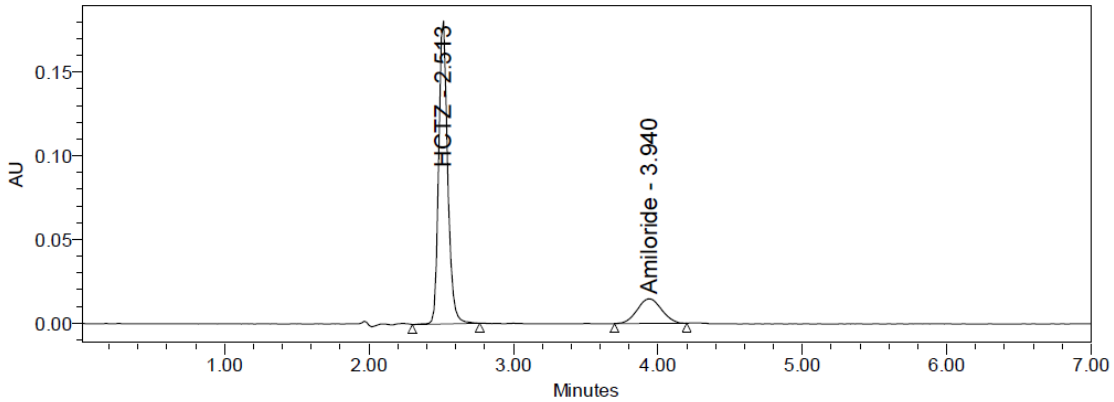

Fig-26- Mobile phase minus Chromatogram of Amiloride and Hydrochlorthiazide method.

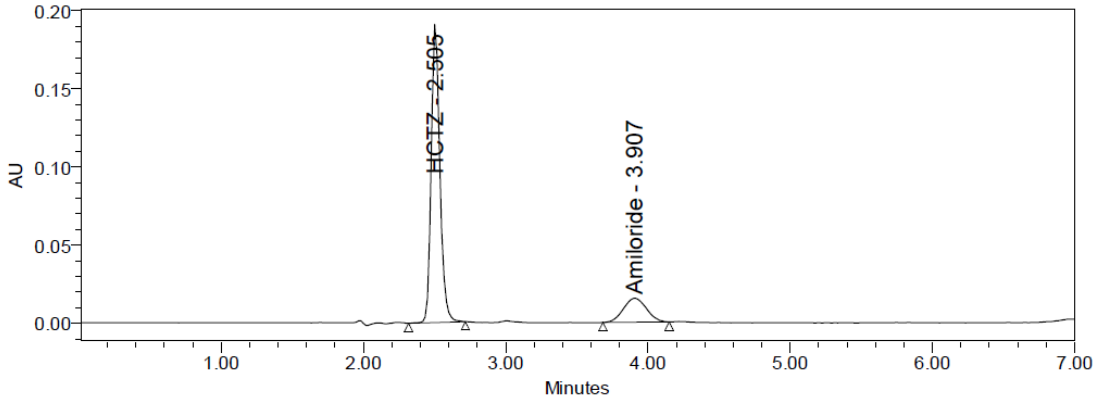

Fig-27-Mobile phase Plus Chromatogram of Amiloride and Hydrochlorthiazide method.

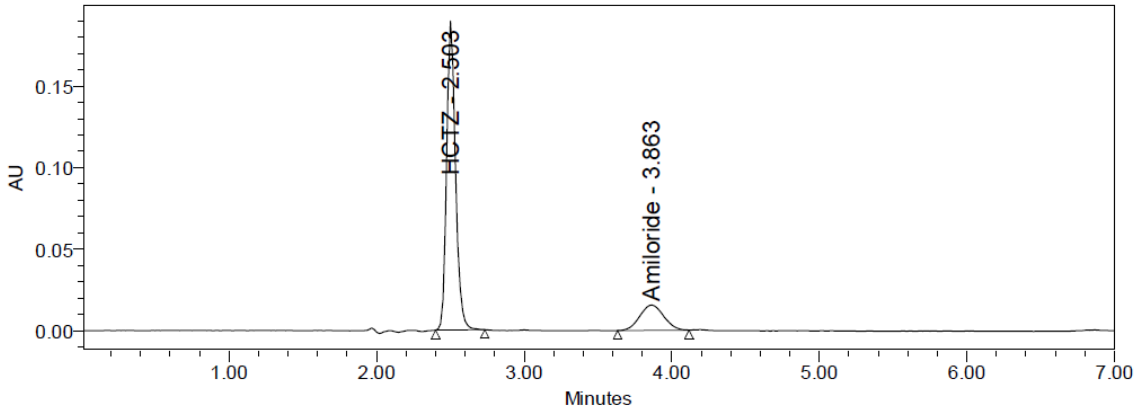

Fig-28- Temperature minus Chromatogram of Amiloride and Hydrochlorthiazide method.

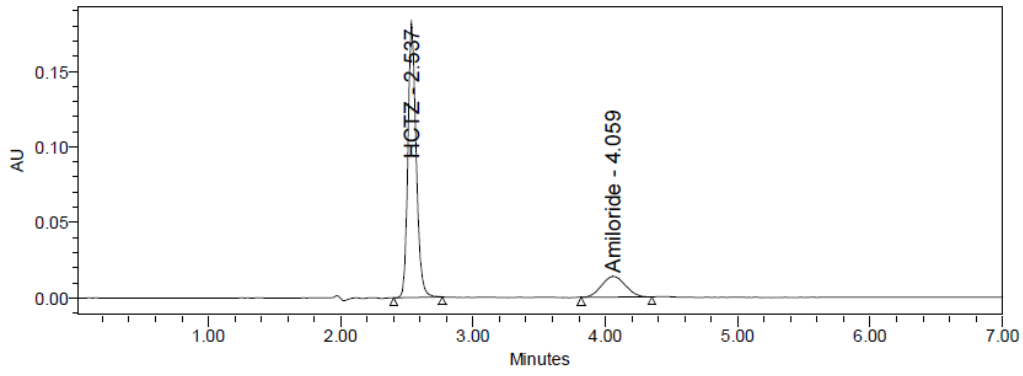

Fig-29- Temperature Plus Chromatogram of Amiloride and Hydrochlorthiazide method.

Assay: Standard preparations are made from the API and Sample Preparations are from Formulation. Both sample and standards are injected six homogeneous samples. Drug in the formulation was estimated by taking the standard as the reference. The Average %Assay was calculated and found to be 100.40 % and 99.9% for Amiloride and Hydrochlorthiazide respectively.

Table-7- Assay of Tablet

S. No.	Amiloride %Assay	Hydrochlorthiazide %Assay
1	99.39	101.23
2	100.40	99.22
3	100.25	100.03
4	100.89	99.57
5	100.44	99.53
6	101.00	100.05
AVG	100.40	99.9
STDEV	0.5748	0.7091
%RSD	0.57	0.71

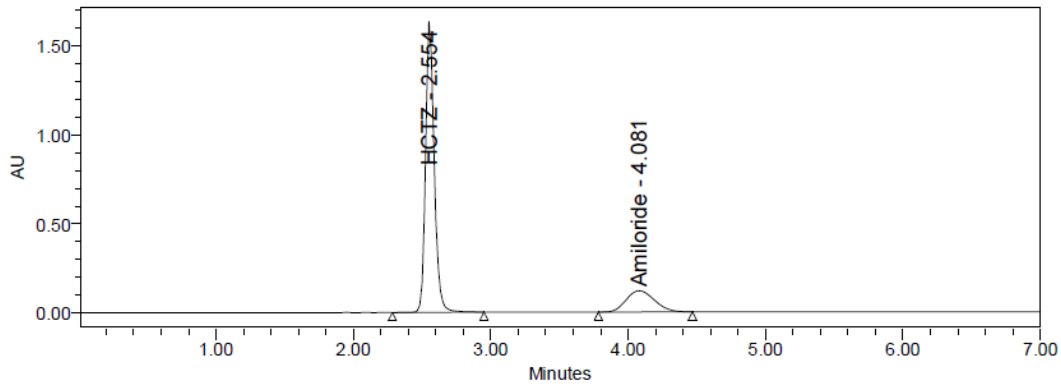

Fig-30-Assay Chromatogram

5. SUMMARY AND CONCLUSION:

Table-8- Summary of work

Parameters	Amiloride	Hydrochlorthiazide
Calibration range (mcg / ml)	5-30 ppm	50-300 ppm
Optimized wavelength	296nm	296nm
Retention time	4.081min	2.554min
Regression equation (Y)	y = 8186x + 1334	y = 3525x + 408.9
Correlation coefficient(r^2)	0.999	0.999
Precision (% RSD*)	0.57	0.71
% Recovery	100.40	99.9
Limit of Detection (µg/ ml)	0.06	0.14
Limit of Quantitation (µg / ml)	0.18	0.41

6. CONCLUSION:

A simple, Accurate, precise method was developed for the simultaneous estimation of the Amiloride and Hydrochlorthiazide in Tablet dosage form. Retention time of Amiloride and Hydrochlorthiazide were found to be 4.081 min and 2.554 min. %RSD of the Amiloride and Hydrochlorthiazide were and found to be 0.57 and 0.71 respectively. %Recover was Obtained as 100.40% and 99.9% for Amiloride and Hydrochlorthiazide. LOD, LOQ values were obtained from regression equations of Amiloride and Hydrochlorthiazide were 0.06ppm, 0.14ppm and 0.18ppm, 0.41ppm respectively. Regression equation of Amiloride is y = 8186x + 1334, and of Hydrochlorthiazide is y = 3525x + 408.9.Retention times are decreased and that run time was decreased so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

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